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Original Article

LEFLUNOMIDE TABLET FORMULATION: DEVELOPMENT AND VALIDATION OF AN RP-HPLC TECHNIQUE

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ABSTRACT

Objective: To develop a Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) method for Leflunomide using rapid, cheap, economical, and less composition of the mobile phase. To validate the method's specificity, linearity, precision, accuracy, robustness, and ruggedness were all validated as per regulatory requirements ICH Q_2 [R1] guidelines.

Methods: The method employed solving of Development and validation based on the measurement of absorbance at one wavelength, 251 nm, λ max, Inertsil-ODS C18 analytical column (250 x 4.6 mm, 5µ). 1.0 ml/min of a mobile phase consisting of water and methanol (40:60v/v) for Leflunomide tablet formulation.

Results: The method showed excellent linear response with correlation coefficient (R^2) values of 0.999 for a Leflunomide. The percent recoveries for a drug were found within the acceptance limit of (99.93%–100.34%). Intra-and inter-day precision studies of the new method were less than the maximum allowable limit percentage of relative standard deviation (%RSD)≤ 2.0. It can be concluded from the results that the present method for validation determination of Leflunomide in tablets is specific, rapid, and simple with good sensitivity.

Conclusion: This analytical method is also applicable in ordinary laboratories and also technique may be used to measure the drug and assess the uniformity and purity of the dosage formulation as well as for quality control of commercial Leflunomide tablets.

Keywords: HPLC, Leflunomide, Validation, Tablet

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INTRODUCTION

Leflunomide [N-[4',5'-trifluoromethylphenyl]-5-methylisoxazole-4-carboxamide] is an isoxazole derivative used as an anti-rheumatic drug with a molecular weight of 270.2 (fig. 1) [1]. The mechanism of action is selective inhibition of dihydro-orotate dehydrogenase [2], a crucial enzyme in the denovo synthesis of pyrimidine, and the subsequent suppression of Ribosenucleic acid and Deoxyribonucleic acid synthesis [3]. Leflunomide may be particularly toxic to activated T cells, which mainly generate pyrimidines through the de novo route [4]. Blockade of tumor necrosis factor is one of the leflunomide's immunomodulatory and anti-inflammatory actions, which have recently been reviewed [5]. Reactive oxygen radicals [6] are inhibited by mediated activation of the transcription factor NFêB. Increases in tissue inhibitor of metalloproteinase (matrix metallo-proteinases) ratios as a result of polymorphonuclear leucocyte movement into the rheumatoid synovial cavity, suppression of matrix metalloproteinases, and patients with its metabolism [7]. Leflunomide in plasma was determined using LC-MS, HPLC, etc. It has been reported in recent research describes the pharmaceutical determination of leflunomide by FIA-UV. Leflunomide in pharmaceutical formulations may be regularly checked for quality using the approach described in this study, which is quick and sensitive and uses UV detection [8] Linearity, accuracy, precision, and robustness were the criteria used to validate the approach. The robustness and intermediate accuracy of the experimental design were validated [9].

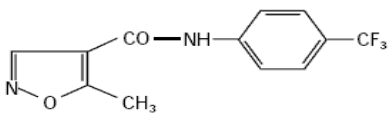

Fig. 1: Structure of leflunomide

Experimental design

Apparatus

HPLC system

Chromatographic separation was achieved using the RP-HPLC Waters system, which includes the Waters Model No. 2690/5 UV-Visible detector, Waters Pump Control Module-II, Waters 515 Solvent Delivery System [10] (pump), Rheodyne-injector (20µ loop), and Waters Empower-2 software from the Waters Corporation as the data processor. Column for analysis Inertsil-ODS C18 (250 x 4.6 mm, 5µ). Spincotech Pvt Ltd's Sonicaultra-sonic cleaner was used to degas the mobile phase after it had been passed through a 0.45 µm membrane filter.

Reagents

Methanol and Acetonitrile of HPLC grade (A. R. grade) were provided from (MerckIndia). Sun Pharmaceuticals Ltd. Baroda, India provided a pure sample of the medication and an internal standard [11]. All solutions for the procedure were made with ultra-pure water made with a Milli-Q® UF-Plus device (Millipore) [12]. Leflunomide in conventional formulations was determined using [lefra®] 20 mg tablets.

Chromatographic condition

By completing several trials using various mobile phases and altering their compositions and flow rates, the chromatographic conditions were eventually optimized, resulting in the development of an optimized chromatogram [13] [table 6].

Preparation of mobile phase

Using a vacuum filtration method, the mobile phases, water and methanol were taken in a 40:60 ratio v/v. Furthermore, the mobile phases were filtered through a membrane filter and sonicated for 15

min in an ultrasonic water bath. (Millipore nylon disc filter 0.45 µm) [14] Before usage [15].

Standard stock and standards solution preparation

10 mg of Leflunomide was accurately weighed and added into a 10 ml volumetric flask, first dissolving it in a required amount of methanol, followed by sonication for 10 min [16]. Methanol should be added after the solution has been made up, obtaining a concentration of 1000, 100, 10 mcg/ml [17].

Extraction of leflunomide from tablets

20 Leflunomide containing lefra® tablets, equivalent to 20 mg, were weighed and added to a 100 ml volumetric flask [1]. 90 ml of methanol was added, and the mixture was centrifuged at 1000 rpm for 30 min after being steeped in an ultrasonication bath for 10 min. To volume, the supernatant was diluted with the same solvent. Furthermore, a 0.45 µm filter was used to filter the solution, and the filtrate was used to make sample solutions in various concentrations [18].

Preparation of calibration curve standards

The concentration range for the calibration curve was 20–70 µcg/ml, and the required amount of mobile phase was added. Through the use of 0.45 µm membrane filter paper, the formed solutions were filtered, and the filtrate was used for analysis [19].

Optimized method development and validation

We have created a quick and accurate RP-HPLC technique for extract sample quantification for this investigation. ODS C18 Column Inertsil [250 x 4.6 mm, 5µ] [20]. Water and methanol were utilized as the mobile phase and column in a ratio of 40:60 v/v. 1.0 ml/min flow rate. The 256 nm wavelength was used for the detecting process. The developed technique the created procedure was precise and accurate [21] [fig. 3].

RESULTS AND DISCUSSION

Leflunomide 10µcg/ml and the internal standard Reslizumab 10 µcg/ml could be separated well using the chromatographic conditions that were used [fig. 2]. No drug deterioration was detected during the analysis. The following measures were used to validate the Liquid chromatography technique [9].

Validation

The optimized chromatographic method was completely validated to the procedures in ICH guidelines validation of analytical methods ICH $Q_{2\,[R1]}$ [22].

Linearity: [n=6]

By using the mobile phase, chromatography was performed on leflunomide and an internal standard [23]. Leflunomide was used to examine the linearity of peak area responses to concentrations from 20 to 70µcg/ml. Over the studied concentration range, a linear response was seen. The results are tabulated in [table 3].

Accuracy and precision

Leflunomide concentrations of 20, 40, and 60 µcg were included in three separate solutions used to determine accuracy [24]. The obtained values were within the range of 99.93%100.24%, 100.34% mean (Relative Standard Deviation) RSD% was 0.00431, satisfying the conditions for the study's acceptance. The reproducibility was determined with five injections of Leflunomide with an analytical concentration of approximately 40 µcg [25]. The RSD% was 0.00352 and 0.00373, respectively [table 2].

Table 1: Data on the accuracy of leflunomide [n=3] where "n"=three different concentration

S. No.	Amount added mcg/ml	Recovery level	Amount recovered mcg/ml	% Recovery [n=3]	%RSD
1	20	50%	19.98	99.93%	0.00342
2	40	100%	40.09	100.24%	0.00431
3	60	150%	60.20	100.34%	0.00145

Values are presented in the form of mean±RSD

Table 2: Leflunomide repeatability data

S. No.	Concentration µg/ml	Intraday [n=3]			Interday [n=3]		
1	40 µcg/ml	I	II	III	I	II	III
		3058687.12	3058588.92	3058782.28	3058349.65	3058594.07	3058580.67
Mean		3058632.20			3058537.12		
SD		107.9303			114.2974		
%RSD		0.00352			0.00373		

n=peak area of three determination, Values are presented in the form of mean±SD

Specificity

In the drug's High-performance liquid chromatography, the chromatograms showed relatively no peaks within a 6 min retention time range. No interference was observed from the additives and by-products. This method was found to be specific [26]. The stability of the stock solution was evaluated under two conditions, at room temperature, stored in the refrigerator (2-8 °C). Consequently, it was determined that the peak is peculiar for this particular Leflunomide [27].

Table 3: Data on the linearity of Leflunomide

A statistical attribute	HPLC
Concentration range (mcg/ml)	20-70
Regression equation	y = 76034x+9579.
Correlation coefficient (r)	R^2 = 0.9999
Slope	76034
y-Intercept	9579
Limit of detection [LOD] (µg/ml)	0.0041
Limit of quantification [LOQ] (µg/ml)	0.0126

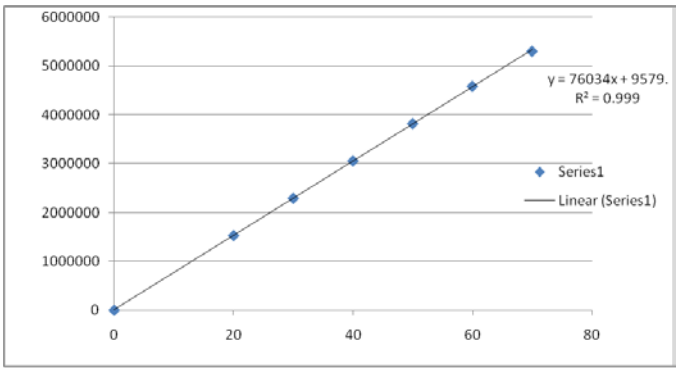

Fig. 2: Plot of linearity (Concentration Vs Peak area) [n=6], n=peak area of six determination, % RSD; Percentage relative standard deviation

Robustness

According to the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use(ICH), an analytical procedure's robustness is its capacity to be unaffected by minor and intentional changes to the method's parameters [ICH, 1997] [28]. In robustness testing, a multivariate strategy incorporating the design of experiments is advised to explore the simultaneous change of the variables on the taken-in responses. According to the system appropriateness criteria, theoretical plates and asymmetry were determined to be in excellent condition [29]. Thus, the investigation supports the validity of the test technique for detecting even little chromatographic condition changes. Thus, the approach may be described as robust [30].

Table 4: Leflunomide robustness study, SD; Standard deviation

Flow rate	STD. peak mean	Tailing factor mean	SD	%RSD
0.8 ml	1924774.87	1.112	87.2224	0.00453
1.0 ml	3058552.29	1.113	127.5140	0.00416
1.2 ml	4132485.35	1.115	134.8511	0.00326

Values are presented in the form of mean±SD

Table 5: Limit of quantification and LIMIT of detection study of leflunomide

Parameter	Criteria	Formula	Results
LOD	S/N = 3	3.3 x S. D/Slope	0.0041µg/ml
LOQ	S/N =10	10 x S. D/Slope	0.0126µg/ml

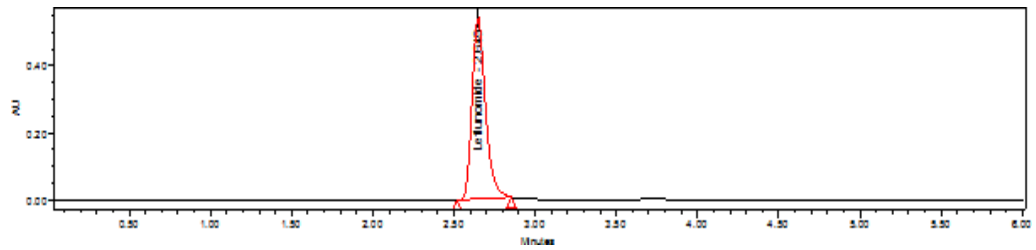

Fig. 3: The leflunomide chromatogram [25 mg]

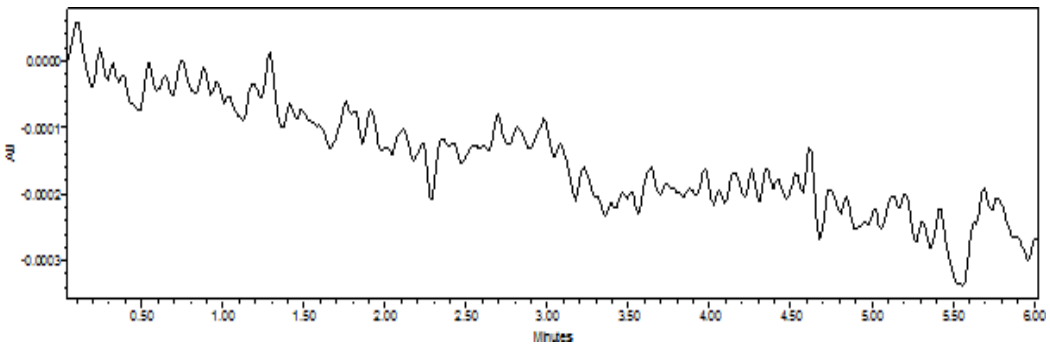

Fig. 4: The leflunomide blank chromatogram

Table 6: Separations trails with different mobile phase compositions on C18 column (ODS)

Trial No	Mobile phase composition	Flow rate	t_R	Remarks
1	Acetonitrile: Water [90:10 v/v]	1.0 ml/min	3.071	A bold Peak was observed
2	Acetonitrile: methanol [45:55 V/V.]	1.0 ml/min	2.831	Got noise baseline, peak shape was not good
3	Acetonitrile: Methanol [50:50 V/V.]	1.0 ml/min	3.336	Peak Tailing was observed.
4	Methanol: Water [60:40 V/V]	1.0 ml/min	2.650	The sharp chromatogram and peak shape were good.

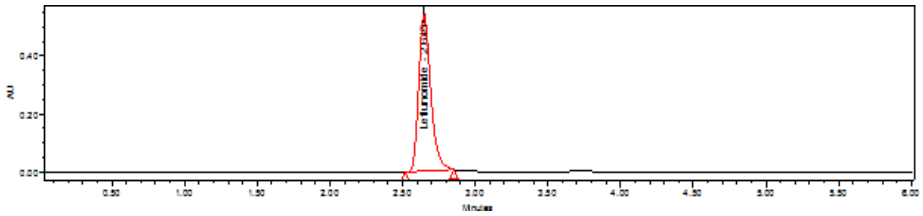

Fig. 5: Chromatogram for leflunomide 40 µcg/ml (Accuracy)

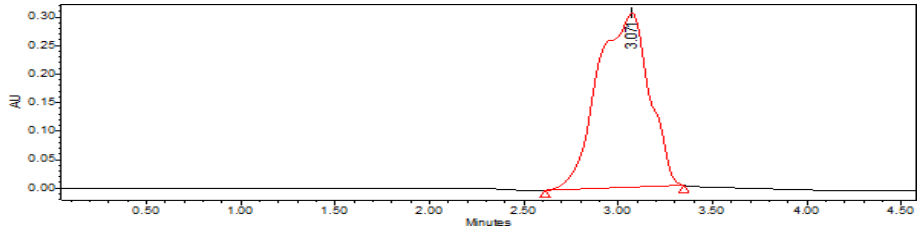

Fig. 6: Trail-1

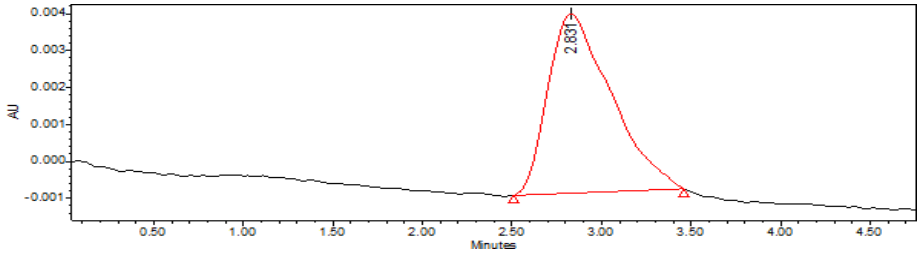

Fig. 7: Trail-2

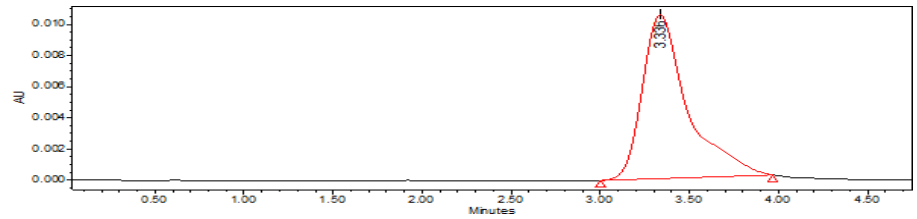

Fig. 8: Trail-3

Table 7: An overview of method validation by reverse-phase high-performance liquid chromatography

Parameters	Leflunomide
Specificity	Peak Purity-No interference (by Uv-PDA detector)
Linearity and Range	20-70 mcg/ml
Regression equation	y = 76034x+9579
Correlation coefficient	0.999
Accuracy-50%	99.93%
100%	100.24%
150%	100.34%
Precision-Intraday	0.00352
Inter day	0.00373
Repeatability	0.00362
LOD	0.0041µg/ml
LOQ	0.0126µg/ml
Robustness	The system suitability parameters were determined to be well within the accepted standards, so that method should be robust

An overview of the conditions for method validation

The whole list of unique validation parameters produced by the Reverse phase-High-performance liquid chromatography technique for the Leflunomide tablet.

CONCLUSION

The suggested high-performance liquid chromatographic technique was assessed for linearity, precision, accuracy, and suitability; it was found to be practical and successful for the quality control of Leflunomide in dosing types for pharmaceuticals. With a correlation value of 0.999, it was demonstrated that the measured signal was exact, accurate, and linear across the concentration range examined (20-70 mcg). Additionally, the chromatographic process is economical and ecologically beneficial due to the minimum solvent consumption and the brief analytical run time of 6.0 min. It is clear from the findings that the suggested approach may be used to determine Leflunomide with reliable sensitivity and without causing any interference. As a result, the proposed methodology is quick, selective, and only needs a quick sample preparation step, and provides a good method for making tablets with Leflunomide.

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Nil

AUTHORS CONTRIBUTIONS

The investigation, original draft, writing, formal analysis, data curation, validation, editing: Pothuraju Naresh, Conceptualization, resources, supervision: Dr. K. Vinod Kumar, All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of this work, ensuring integrity and accuracy. All authors have read and agreed to the published version of the manuscript.

CONFLICT OF INTERESTS

No conflicts of interest are reported by the authors.

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